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Unfractionated Heparin and Low Molecular Weight Heparin do not Inhibit the Growth of Proliferating Human Arterial Smooth Muscle Cells in Culture

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Objectives: *To clarify the effects of unfractionated heparin (UH) and low molecular weight heparin (LMWH) on proliferating human smooth muscle cells (SMC) compared to growth arrested SMC.*
Design: *A cell culture study where proliferating SMC were exposed to different concentrations of UH and LMWH and the effect on proliferation and collagen secretion was studied. Growth arrested SMC were stimulated with serum and the effect of UH on proliferation was measured.*
Setting: *Sections of Medical Angiology and Vascular Surgery, Malmö General Hospital, Sweden.*
Materials: *Human SMC were established from arterial tissue obtained at vascular surgery or at organ donation.*
Chief outcome measures: *Effects of UH and LMWH on total cellular DNA, ^{3}H-thymidine incorporation and collagen secretion using proliferating and growth arrested human SMC in culture.*
Main results: *In proliferating SMC that had not been growth arrested, 1 and 10 IU/ml UH and LMWH significantly increased total cellular DNA compared to controls while DNA synthesis was not influenced. The higher cellular DNA was probably not a consequence of increased proliferation as DNA synthesis was not affected by UH or LMWH. The increased total cellular DNA could instead be due to reduced cell death. Higher concentrations (10 IU/ml) of UH and LMWH also increased collagen secretion. In control experiments with UH DNA, synthesis was decreased in stimulated human SMC that had been growth arrested previously to heparin exposure.*
Conclusions: *The effects of UH and LMWH on SMC proliferation will depend on the proliferative state of the SMC. The results might be of relevance for the understanding of the atherosclerotic process and for pharmacologic interventions to prevent restenosis after angioplasty or surgery.*

Key Words: Smooth muscle cells; Proliferation; Collagen; Heparin; Atherosclerosis.

Introduction

Heparin is an effective inhibitor of SMC proliferation and migration both *in vivo* and *in vitro*.[1–3] Furthermore, heparin influences the composition of the cellular matrix. *In vivo* elastin and collagen are reduced while proteoglycans are increased.[4] The mechanism behind these effects of heparin have not been clarified, but the inhibitory effect on cell proliferation seems to include rapid internalisation through high affinity receptors[5] and intracellular metabolism of heparin.[6]

Heparin has been shown to reduce intimal thickening after arterial injury in animal models.[7] These data have provided evidence that heparin treatment must be started within the first 24 h after injury to be effective.[8] However, in man, heparin does not prevent myointimal hyperplasia in vein grafts[9] and 18–24 h of heparin administration after coronary angioplasty did not decrease the incidence of late restenosis.[10] In the normal, uninjured arterial wall, almost all SMC are in the non-proliferating (growth-arrested) state while a substantial proportion of the cells are in the proliferating state after different forms of arterial injury.[11] The fibrous cap of the atherosclerotic plaque is made up of a subintimal proliferation of SMC that also have synthesised the extracellular matrix surrounding them.[12] In most *in vivo* and *in vitro* models concerned with the effect of heparin on SMC proliferation, effects after stimulation of growth arrested cells have been studied.

The fact that heparin has not been shown to inhibit SMC proliferation in humans, i.e. restenosis after

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angioplasty, led us to compare the effects of UH and LMWH on growth parameters of human SMC in different proliferative state *in vitro*.

Materials and Methods

Cell culture

Segments of different arteries were obtained at vascular surgery ($n = 19$) or from organ donors ($n = 5$) (ethical approval was obtained from the Swedish Social Welfare Board). The mesenteric, iliac and femoral arteries were used as well as pieces from the thoracic and abdominal aorta. The arteries were immediately put into 0.15 mol/l saline (4°C) for a maximum of 8 h until cultures were established.

Explants from medial segments of the arteries were used for the establishment of cultures as previously described.[13] Cells were grown in minimal essential medium (MEM, Gibco), Hepes buffered 25 mmol/l, supplemented with 10% fetal bovine serum (FBS) (KEBO, heat inactivated) and 10% human serum (HS) (obtained from the blood bank), L-glutamine 585 mg/l, benzylpenicillin 150 μg/ml and streptomycin 100 μg/ml. Subcultures (1:2 split) were obtained by the use of trypsin 0.05%-EDTA 0.02%.

Confluent cells from passage two to five were trypsinised and counted, the cell suspension was then transferred to multiwell dishes (NUNC, 24 wells/dish). Each well contained 3–4 $\times$ 10^4 cells in 1 ml medium from the start, and growth was established during 24 h. After this period cell layers were 30–50% confluent.

Experimental procedure

Unfractionated heparin (5000 IU/ml) and low molecular weight heparin (Fragmin^R) (2500 IU/ml) were obtained from Kabi-Pharmacia and diluted with MEM to obtain a concentration of 1 and 10 IU/ml for both UH and LMWH. The lower concentration corresponds to that obtained with heparin treatment in a clinical situation.

The medium was changed to MEM, with 5% FBS and 5% HS, and 200 μl of the standard solutions of UH and LMWH were added. Controls received 200 μl serum free (SF) MEM. The cultures were incubated for 72 h. Total cellular DNA was measured from the start and after 72 h, ^{3}H-thymidine incorporation between 20 and 24 h and collagen between 24 and 72 h.

Human SMC were growth arrested in SF MEM for 24 h whereafter fresh medium with 5% FBS/5% HS was added together with UH. ^{3}H-thymidine incorporation was assayed between 20 and 24 h. ^{3}H-thymidine incorporation at the start of the experiments (O h) was 150–300 dpm/dish for growth arrested cells and 1.087–19.757 dpm/dish for proliferating human SMC.

Measurements

To measure DNA synthesis, 0.0033 μCi/ml medium of methyl-(^{3}H)-thymidine (Amersham, specific activity 25 Ci/mmol) was added to each dish after 20 h incubation. Four hours later ^{3}H-activity was determined as previously described.[13]

Cells were scraped with a teflon policeman from each dish to determine total cellular DNA.[14]

Cells were labelled with 5-^{3}H-proline (1.25 μCi/ml) (Amersham) during 48 h. The newly synthesised ^{3}H-labelled collagen was determined after pepsin digestion and successive salt precipitations at acid and neutral pH in the presence of carrier collagen as described by Webster and Harvey.[15]

Cell counting was performed in a Burker haemocytometer chamber and radioactivity counted in a Tri-Carb 300 CD liquid scintillation spectrometer. The Wilcoxon test for paired data was used for the comparisons.

Results

Proliferating cells

No significant differences in DNA synthesis between controls and cells exposed to 1 or 10 IU/ml UH or

Table 1. Effects of UH and LMWH on DNA synthesis of proliferating human SMC

Concentration	Control	UH	LMWH
1 IU/ml (n=24)	4469*† (2039–5639)	3559*† (2306–5461)	3683† (2124–5304)
10 IU/ml (n=18)	5039‡§ (3235–10129)	5028‡§ (2686–9946)	4457§ (3284–9540)

*$_{NS}$; †$_{NS}$; ‡$_{NS}$; §$_{NS}$

The values represent ^{3}H–thymidine incorporation (dpm/dish). Results are presented as median value with interquartile range, the latter in parentheses. Triplicate dishes were used. n=number of experiments. ^{3}H–thymidine incorporation was assayed after 20–24 h incubation.

LMWH were found (Table 1). The supply of cells in some experiments was insufficient which explains the somewhat smaller number of experiments with the higher concentration.

Table 2. Effects of UH and LMWH on total cellular DNA of proliferating human SMC

Concentration	Start	Control	UH	LMWH
1 IU/ml (n=24)	0.47 (0.35–0.52)	0.63*† (0.58–0.78)	0.70*† (0.64–0.83)	0.73† (0.60–0.86)
10 IU/ml (n=18)	0.46 (0.36–0.51)	0.65‡§ (0.60–0.73)	0.72‡§ (0.58–0.80)	0.75§ (0.61–0.79)

*$p < 0.005$; †$p < 0.005$; ‡$p < 0.05$; §$p < 0.05$
The values represent μg DNA/dish. The results are presented as median value with interquartile range. Conditions otherwise identical to Table 1. DNA was measured at start and after 72 h incubation.

The results for total cellular DNA are shown in Table 2. The clear increase of cellular DNA in controls between start and 72 h indicated that the cells were proliferating. A concentration of 1 IU/ml UH or LMWH caused an increase in total cellular DNA compared to controls ($p < 0.005$). For 10 IU/ml of UH and LMWH similar results were obtained ($p < 0.05$). A concentration of 1 IU/ml UH and LMWH did not influence collagen secretion (Table 3) while 10 IU/ml UH and LMWH increased collagen secretion ($p < 0.05$ and 0.005 respectively).

Table 3. Effects of UH and LMWH on secretion of newly synthesised collagen by proliferating human SMC

Concentration	Control	UH	LMWH
1 IU/ml (n=24)	2580*† (1908–3893)	2413*† (1716–4816)	2600† (1821–4452)
10 IU/ml (n=18)	2975‡§ (2065–5683)	3889‡§ (1864–6342)	5468§ (3599–7868)

*NS; †NS; ‡$p < 0.05$; §$p < 0.05$
The values represent collagen secretion (dpm/dish) per total cellular DNA (dpm/μg). Experimental conditions as in Tables 1 and 2. Collagen was assayed at 24–72 h.

Growth arrested cells

In six experiments with human SMC, ^{3}H-thymidine incorporation was measured in cells that were stimulated with serum after they had been growth arrested (Table 4). In parallel cultures, proliferating SMC were grown as previously described. Under these conditions, 1 and 10 IU/ml UH inhibited DNA synthesis by 27–34% in stimulated, previously growth arrested cells ($p < 0.05$) while proliferating cells were not inhibited.

Table 4. Effects of UH on DNA synthesis of proliferating and stimulated growth arrested human SMC

Concentration	Growth arrested SMC (n=6)	Proliferating SMC (n=6)
Control	5683*† (5238–6436)	4264*§ (4006–4532)
1 IU/ml	4159*† (3867–4676)	4238‡§ (3998–4459)
10 IU/ml	3750† (3145–4218)	4158§ (3879–4458)

*$p < 0.05$; † $p < 0.05$; ‡NS; §NS
Parallel experiments were performed with the same cell population and passage comparing proliferating and previously growth arrested cells. The values show ^{3}H-thymidine incorporation/dish. Each value represents mean of six identical experiments using triplet dishes, the interquartile range is given within parentheses. DNA synthesis was measured between 20–24 h of incubation.

Discussion

Heparin and heparin-like molecules are well established inhibitors of SMC proliferation, but the vast majority of experiments, both *in vivo* and *in vitro*, have been performed with growth-arrested cells that were stimulated by arterial wall injury and/or addition of growth factors.[1–3] In the present study we have used proliferating human SMC and compared the effects of UH and LMWH to those on growth-arrested SMC that were growth stimulated with serum. The main finding was that proliferating cells were not growth inhibited by UH or LMWH in our model. Recently, Caplice *et al.* have reported that the proliferative state of the cells involved in outgrowth from coronary artery explants may influence the activity of heparin.[16] Their findings were quite similar to ours. Furthermore, and in contrast to previous studies,[4] we found that collagen secretion was not influenced (1 IU/ml UH and LMWH), or was increased (10 IU/ml UH and LMWH).

For comparison, human SMC were growth arrested and then stimulated by the addition of serum. The expected inhibition of proliferation by UH was obtained, thus ruling out the possibility that the drug was pharmacologically inactive in our models.

The human SMC were obtained from both normal arterial segments (no macroscopic atherosclerosis) and from pieces affected by atherosclerosis. No differences between cells derived from these different segments

with respect to the reaction to UH or LMWH could be detected. Furthermore, there were no differences between cells from different arteries concerning the parameters studied. Therefore all cells could be regarded as similar. SMC from organ donors were generally derived from less atherosclerotic segments than those from vascular surgery patients. The organ donors were also, in most cases, younger than the surgical patients. Growth was established from approximately 60% of the vascular surgery tissue and 90% of organ donors. The latter SMC could generally be propagated to the fifth or sixth passage while the fourth passage was seldom reached with cells from atherosclerotic tissue.

^{3}H-thymidine incorporation was analysed after 24 h while cellular DNA and collagen secretion was determined after 72 h. The reason for this was that the ^{3}H-thymidine method will detect differences in DNA synthesis within the first 24 h after stimulation, corresponding roughly to one cell cycle. In contrast, longer incubation time is needed to detect differences in cellular DNA or collagen secretion.

We used 24 h in SF MEM to obtain growth arrest. Periods longer than 24 h will result in extensive cell detachment and will not decrease the ^{3}H-thymidine count further. Therefore we considered the cells after 24 h in SF MEM to be growth arrested. Cell detachment within 24 h was 10–20%.

UH and LMWH increased total cellular DNA compared to controls. Total cellular DNA is a good parameter for cell number and, in our experience, better than cell counting. The cell number is a balance between cell proliferation and cell death, and therefore the increased DNA values after exposure to UH and LMWH were not necessarily due to stimulation of SMC proliferation. As ^{3}H-thymidine incorporation did not differ between the different groups, a possible interpretation would be that different heparins may reduce cell death. There is some support for this explanation in the literature.[17] Other reports have shown that heparin will potentiate the effect of some growth factors.[18,19] Similarly, the effects on collagen secretion may be a consequence of reduced cell death, but the clear increase, at least with 10 IU/ml LMWH also suggests some direct stimulating effect.

The results obtained stress the importance of clearly identifying whether effects of UH or LMWH are studied on proliferating or growth arrested SMC as the results may be quite different. The results may also explain why the inhibiting effects of heparin on SMC proliferation shown in animal models and *in vitro* are not always as clear when studied in more clinical situations, for example prevention of restenosis after angioplasty or suppression of intimal proliferation in vein grafts.[9] In these models it is not evident that the majority of cells are growth arrested, as traumatisation of the arterial wall could be caused by the operative procedures or the atherosclerotic process.

We conclude that UH and LMWH do not inhibit proliferation of proliferating human SMC, in contrast to previously stimulated growth arrested SMC. Instead, there were indications that UH and LMWH may reduce cell death of proliferating SMC *in vitro* as well as supporting collagen secretion. The results may be of importance for the understanding of pharmacological intervention of uncontrolled SMC proliferation.

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